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Review article

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Cellular stress in the pathogenesis of nonalcoholic steatohepatitis and liver fibrosis

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Abstract

The burden of chronic liver disease is rising substantially worldwide. Fibrosis, characterized by excessive deposition of extracellular matrix proteins, is the common pathway leading to cirrhosis, and limited treatment options are available. There is increasing evidence suggesting the role of cellular stress responses contributing to fibrogenesis. This Review provides an overview of studies that analyse the role of cellular stress in different cell types involved in fibrogenesis, including hepatocytes, hepatic stellate cells, liver sinusoidal endothelial cells and macrophages.

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Key points

- Chronic activation of endoplasmic reticulum stress in hepatocytes drives nonalcoholic steatohepatitis (NASH) pathogenesis, insulin resistance, fat accumulation, inflammation, fibrogenesis and liver fibrosis through inadequate unfolded protein response, cell death and inflammation.
- Mitochondrial dysfunction and oxidative stress drive NASH and liver fibrosis by causing hepatocyte damage, immune cell activation and inflammation.
- Cellular stress pathways in hepatocytes also contribute to fibrogenesis independent of NASH.
- Intracellular reactive oxygen species generation provokes endoplasmic reticulum stress and autophagy that contribute to the activation of hepatic stellate cells.

Introduction

The global burden of chronic liver disease is rising: cirrhosis, or end-stage liver disease, is the 11th leading cause of death and the 14th leading cause of morbidity worldwide[1]. Hepatic fibrosis is the common pathological mechanism that leads to cirrhosis and occurs following chronic liver injury from toxins (alcohol), infections (hepatitis B virus (HBV) and hepatitis C virus (HCV)) and metabolic disease (nonalcoholic fatty liver disease (NAFLD), or, as is currently proposed, metabolic dysfunction-associated steatotic liver disease[2]). Hepatic fibrosis is characterized by the deposition and crosslinking of extracellular matrix proteins[3]. Clinical studies have demonstrated that hepatic fibrosis is reversible in some patients; for example, successful treatment of HCV infection has resulted in fibrosis regression[4]. However, there are limited therapeutics that target hepatic fibrosis, and organ transplantation is available for a minority of patients.

There is increasing evidence suggesting that cellular stress and maladaptive responses contribute to fibrogenesis. Understanding the molecular mechanisms of cellular stress leading to fibrogenesis could identify new therapeutic targets to halt disease progression and address a large unmet medical need. This Review summarizes current insights into cellular stress in fibrogenesis according to cell type, focusing on hepatocytes, hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs) and macrophages.

Cell types in hepatic fibrosis

The pathogenesis of hepatic fibrosis includes an interplay of diverse cell types, including hepatocytes, inflammatory cells, LSECs and HSCs (Fig. 1). Prior in vivo and in vitro studies have revealed key molecular mechanisms and are discussed in detail in a review article published in 2021 (ref. 3). In response to injury, hepatocytes express factors such as NOTCH, osteopontin, transforming growth factor-β (TGFβ), NADPH oxidase 4 (NOX4), TAZ and Indian Hedgehog protein. Expression of these factors triggers an inflammatory response, leading to macrophage activation, the release of reactive oxygen species (ROS) and TGFβ, and the activation of quiescent HSCs into myofibroblasts[3]. Activated HSCs migrate to and proliferate at sites of injury and produce extracellular matrix proteins, including type I collagen, leading to the formation of the fibrous scar[5,6]. Importantly, single-cell RNA sequencing techniques have elucidated heterogeneity within cell types in the context of fibrosis[7].

Cellular stress pathways in hepatocytes and NAFLD progression

Cellular stress pathways have been studied in detail in the context of hepatocytes and the pathogenesis of NAFLD[8]. We first discuss the mechanisms of hepatocyte cellular stress in NAFLD and nonalcoholic steatohepatitis (NASH), or, as is currently proposed, metabolic dysfunction-associated steatohepatitis[2], before specifically addressing liver fibrosis. This approach was chosen because of the importance of NASH as a primary cause of liver fibrosis and the growing evidence suggesting that hepatocyte cellular stresses in NASH might directly contribute to fibrogenesis. Figure 2 highlights the intricate network of cellular stresses, including endoplasmic reticulum (ER) stress and mitochondrial dysfunction, and the activated signalling pathways involved in NASH, underscoring their role in hepatocyte injury, inflammation and disease progression.

Canonical endoplasmic reticulum stress pathway

The ER performs crucial functions necessary for maintaining cellular homeostasis and acts as a cellular sensor. It has various major roles, such as the assembly and transport of proteins, the biosynthesis of most lipids and steroids, calcium storage and homeostasis, and the detoxification of cells[9].

When ER function is disrupted by an accumulation of misfolded proteins or lipids or a change in the intracellular calcium concentration, its homeostasis is altered, resulting in ER stress[10]. In response to ER stress, the unfolded protein response (UPR) signalling is activated to increase protein folding capacity and restore cellular homeostasis by decreasing global protein synthesis and activating the transcription of ER-resident proteins required for protein folding[11,12]. However, depending on the intensity and persistence of ER stress, UPR can result in either cell adaptation (adaptive UPR) or cell death by apoptosis (pro-apoptotic UPR)[13]. Three ER transmembrane protein stress sensors are involved in UPR signalling, namely inositol-requiring enzyme 1 (IRE1), protein kinase R-like ER kinase (PERK; also known as EIF2AK3) and activating transcription factor 6 (ATF6)[13]. During ER stress, the chaperone-binding immunoglobulin protein (BiP; also known as GRP78 or HSPA5) dissociates from the three sensors, leading to their activation[14]. UPR signalling pathways promote the expression of genes encoding functions that mitigate ER stress[11,12]. PERK activation leads to a rapid decrease in the mRNA translation and a reduction in the functional load on the ER of new proteins[14]. Although PERK activation reduces general protein synthesis temporarily, activation of activating transcription factor 4 (ATF4) is upregulated and promotes the expression of genes contributing to protein folding, amino acid metabolism, autophagy, antioxidant response and apoptosis by activating pro-apoptotic genes such as *CHOP* (also known as *DDIT3*)[15–17]. IRE1 and ATF6 UPR pathways promote protein folding, transport and degradation[11,12]. IRE1 promotes the splicing of the *XBP1* mRNA to induce translation of the active X-box binding protein 1 (XBP1) transcription factor and transactivation of ER chaperones and ER secretory genes[18,19]. IRE1 also recruits the adaptor protein TRAF2 and phosphorylates the ASK1 to activate the JUN amino-terminal kinase (JNK) pathway. ATF6 promotes a transcriptional response that increases protein folding and ER-associated degradation (ERAD) pathway, similar to IRE1–XBP1 transcriptional activation[11,20–22].

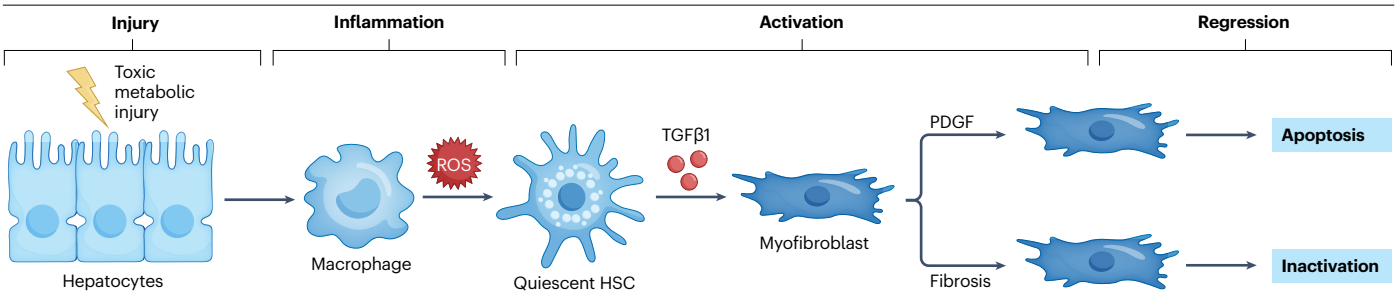

Fig. 1 | Overview of cell types in hepatic fibrosis. Several cell types play a key role in the pathogenesis of hepatic fibrosis. In response to liver injury, hepatocyte damage induces inflammation, leading to macrophage activation, the release of reactive oxygen species (ROS) and transforming growth factor-β1 (TGFβ1), and the activation of quiescent hepatic stellate cells (HSCs) into myofibroblasts. Activated HSCs proliferate in response to various cytokines, including platelet-derived growth factor (PDGF), secrete collagen type I, and induce fibrosis. Following cessation of liver injury, myofibroblasts undergo apoptosis or inactivation, leading to fibrosis regression. Adapted from ref. 3, Springer Nature Limited.

Activation of the three branches of the UPR (XBP1, ATF4 and ATF6) enables the UPR pathway-driven transcriptional response, producing various ER proteins that promote protein maturation. Furthermore, these three transcription factors have a role in activating the ERAD pathway, which facilitates proteasomal degradation and autophagy, a process that eliminates misfolded proteins[23]. Importantly, apoptosis can occur if UPR pathways cannot restore normal ER homeostasis, which is triggered by the activation of pro-apoptotic proteins such as CHOP (PERK pathway) or JNK (IRE1 pathway)[13]. This pro-apoptotic process exemplifies the crosstalk between the pathways involved in the UPR. The controlled activation among these three UPR pathways is intended to reduce ER stress. Although the fundamental aim of the UPR is to restore ER protein homeostasis to ensure cell survival, prolonged activation resulting from uncontrolled severe ER stress favours apoptosis and cell death, which occurs in the setting of NAFLD[9,10,24].

Endoplasmic reticulum stress in NASH

Chronic UPR has been demonstrated to have a crucial role in the pathogenesis of NAFLD by contributing to the development of insulin resistance and fat accumulation in the liver, as shown in mice and humans[10,25,26]. In this section, we highlight the role of hepatocyte ER stress in the pathogenesis of NASH and its substantial implications in driving fibrosis. The chronic activation of ER stress causes UPR to become ineffective and drives key features of progressive NASH, such as cell death, inflammation and fibrogenesis[16,23,27–29]. ER stress activation has been observed in animal models of steatosis and NASH and in patients with steatosis or NASH[30,31]. Puri and colleagues showed variable degrees of UPR activation in the liver of patients with NAFLD ($n = 21$) or NASH ($n = 21$) compared with subjects with healthy liver histology ($n = 17$); the upstream effector of the PERK pathway, eIF2α, was phosphorylated and activated, whereas the downstream effectors ATF4, CHOP and GADD34 were not consistently upregulated[31].

The enhanced expression of ER stress markers, specifically CHOP and BiP, in liver biopsy samples from individuals with NASH ($n = 23$) compared with healthy liver samples ($n = 34$) provides evidence that ER stress-induced apoptosis in liver cells might contribute to the progression of NASH[32]. A study in patients with NASH ($n = 9$) also demonstrated an association between CHOP expression, the NASH activity score (NAS) and the extent of liver injury[33]. As demonstrated by the same group using mouse models and liver biopsy samples from patients, IRE1 RNase activity has a central role in NASH development[34]. Mice deficient in Bax inhibitor 1, an evolutionarily conserved ER membrane protein, fed a high-fat diet (HFD) developed NASH features, namely apoptosis, necroptosis and inflammation, which were attenuated with pharmacological inhibition of the RNase activity of IRE1 (ref. 34). Surprisingly, hepatocyte-specific deletion of *Xbp1*, the direct downstream target of the IRE1 branch of the UPR, induced lipid accumulation, cell death and fibrosis in mice fed a fructose-enriched diet[29]. The susceptibility of *Xbp1*-deficient mice to liver disease was linked to the compensatory upregulation of IRE1 (ref. 29). This observation was confirmed by liver-specific deletion of *Xbp1* and *Ire1*, which protected mice from the injury observed in mice lacking *Xbp1* alone[29]. These findings emphasize a central role in the RNase activity of IRE1 in NASH development[35].

Lipotoxicity. Emerging evidence highlights the effect of saturated free fatty acids (FFAs), such as palmitic acid and stearic acid, on the induction of ER stress and subsequent hepatocyte apoptosis. In patients with NASH ($n = 6$), serum FFAs increase and cause more liver damage than triglycerides[36]. Mouse models have provided valuable insights into the role of saturated FFAs in promoting ER stress and hepatocyte apoptosis. For instance, the reduction in triglyceride formation in *Dgat2*$^{-/-}$ mice fed a methionine–choline-deficient diet (MCD) led to an increase in hepatic FFA content, CYP2E1 protein levels, hepatic lipid peroxidation and oxidative stress markers, lobular necroinflammation and fibrosis[37]. Human studies showed that saturated FFAs, such as palmitic acid or stearic acid, were more abundant in liver tissue in the progression of NASH and activated ER stress-dependent apoptosis in hepatocytes[38]. In addition, in a rat model fed a HFD, increased expression of the ER stress marker CHOP and activated caspase 3 were observed in the liver[39]. These findings further support the notion that saturated FFAs contribute to ER stress-mediated hepatocyte apoptosis. Also, in hepatocyte cell lines, exposure to palmitic acid activated PERK and IRE1 pathways, resulting in the upregulation of CHOP[28]. This induction of CHOP promoted the expression of the death receptor DR5, activating caspase 8 and promoting translocation of truncated BID to mitochondria, initiating the intrinsic apoptotic pathway[27]. Moreover, in vitro human and mouse studies demonstrated that palmitic acid activates the IRE1 pathway, triggering the production of BH-3 family proteins such as PUMA and BIM via JNK and/or CHOP activation, further promoting cytochrome *c* release from mitochondria and triggering mitochondria-mediated apoptosis[40]. By further investigating the complex link between lipotoxicity, ER stress and hepatocyte cell

death in animal models, a deeper understanding of the underlying mechanisms can be gained.

Inflammation. Research data highlight the association between ER stress, the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and pyroptosis in hepatocytes, with liver inflammation and fibrosis in NASH. The NLRP3 inflammasome and its downstream mediators caspase 1 and IL-1β, have been identified as regulators of liver inflammation and fibrosis in NAFLD[41,42]. ER stress has been implicated in the activation of the NLRP3 inflammasome in several human and mouse studies[33,43–45]. A study showed that ER stress induced by lipopolysaccharide (LPS) or tunicamycin in livers from *ob/ob* mice resulted in a transient disease state resembling NASH, accompanied by pyroptotic cell death in hepatocytes[33]. This study clarifies the way hepatocyte ER stress triggers NLRP3 inflammasome: LPS and tunicamycin activated IRE1 and PERK, resulting in CHOP upregulation and hepatocyte apoptosis as well as NLRP3 inflammasome activation (caspase 11, caspase 1 and IL-1β), which ultimately lead to the induction of pyroptosis[33]. Pyroptosis, described further in the next paragraph, enables the transmission of inflammation and inflammasome signals to adjacent cells, including hepatocytes, HSCs and Kupffer cells[46]. Importantly, in this study, tauroursodeoxycholic acid, an ER stress inhibitor, protected mice from NLRP3 inflammasome activation and apoptosis, suggesting a marked relationship between ER stress and NLRP3 activation[33]. This study suggests that CHOP has a crucial role in linking ER stress-induced cell death and NLRP3 inflammasome activation in hepatocytes, therefore

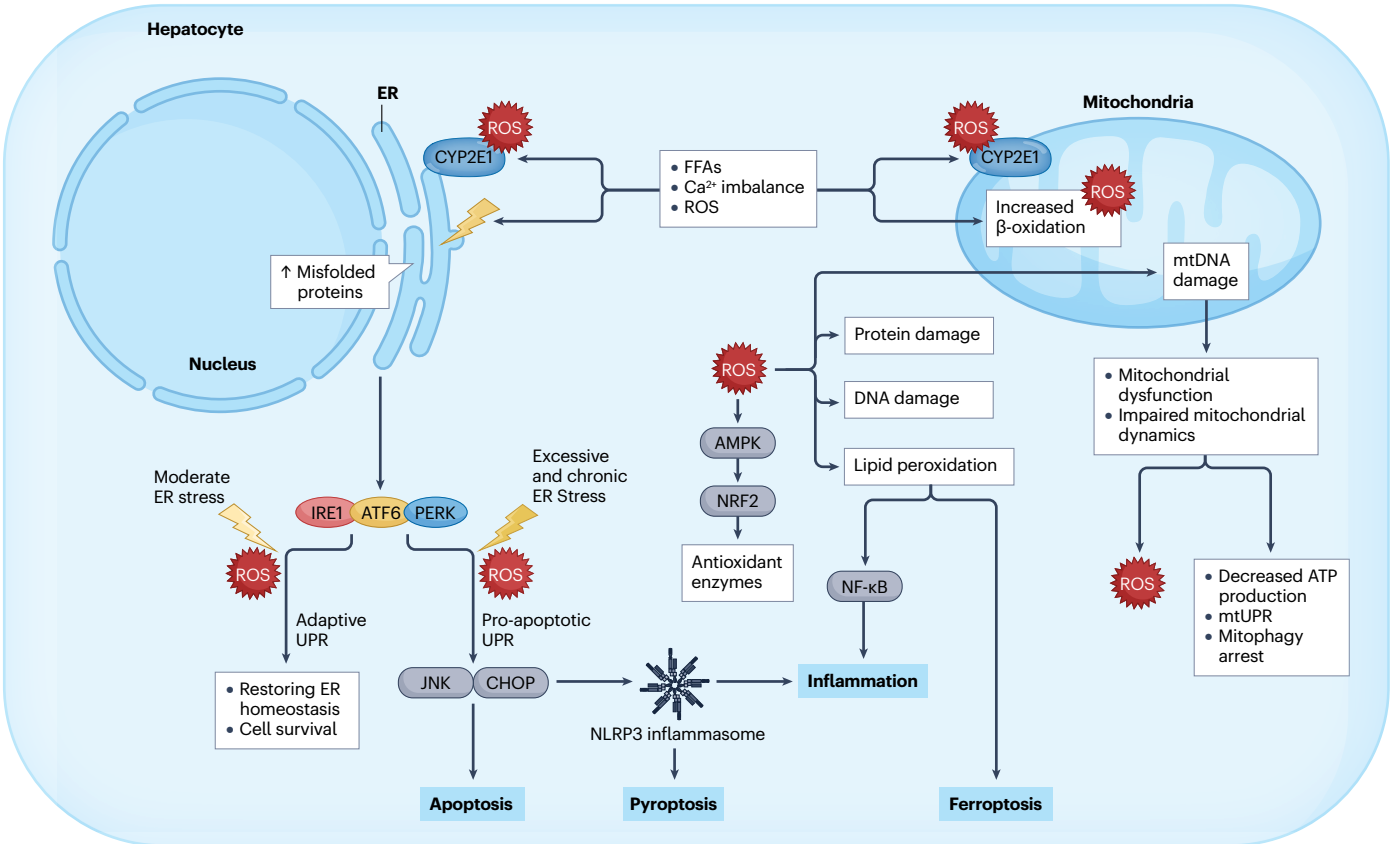

Fig. 2 | Overview of oxidative stress-activated pathways in NASH. In nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), increased entry of free fatty acids (FFAs) into hepatocytes and imbalance of calcium or reactive oxygen species (ROS) levels can lead to moderate endoplasmic reticulum (ER) stress, activating the adaptive unfolded protein response (UPR)[10]. The adaptive UPR aims to restore ER homeostasis and promote cell survival[11,12]. However, in case of excessive and chronic stress, the UPR pathway can become pro-apoptotic, activating pathways such as the JUN amino-terminal kinase (JNK) pathway[13]. Also, activation of C/EBP homologous protein (CHOP) in the pro-apoptotic UPR pathway can trigger the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, promoting inflammation and pyroptosis[33,44,45,48–51]. On the other hand, increased FFA uptake in hepatocytes during NAFLD and NASH enhances β-oxidation in the mitochondria, resulting in increased ROS generation. ROS can cause mitochondrial DNA (mtDNA), nuclear DNA, protein damage and lipid peroxidation[54,55,70,88,89,94–98,100–103]. ROS stimulate AMP-activated protein kinase (AMPK), which, in turn, activates nuclear factor erythroid 2-related factor 2 (NRF2), leading to the expression of antioxidant response genes[66–68,128]. Products of lipid peroxidation activate the nuclear factor-κB (NF-κB) pathway, driving inflammation and promoting ferroptosis[125–127]. Mitochondria-produced ROS can also induce ER stress, activate the JNK pathway and trigger apoptosis[87]. Mitochondrial dysfunction observed in NASH impairs mitochondrial dynamics, further increasing ROS production and decreasing ATP production[72,90,91]. Mitochondrial dysfunction can also lead to the mitochondrial UPR (mtUPR) pathway and impaired mitophagy[134,135]. Additionally, FFAs can be metabolized by cytochrome P450 2E1 (CYP2E1) present in both the ER and mitochondria, leading to increased ROS generation[106–109,111–115]. ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, protein kinase R-like ER kinase.

contributing to the pathogenesis of NASH. Furthermore, although research on the role of the NLRP3 inflammasome in liver disease pathogenesis has received the most attention, current studies have started investigating the potential contributions of other inflammasomes, such as NLRP1, NLRP6, NLRC4 and AIM2 in NASH development[47]. However, whether hepatocyte-specific ER stress is involved in the activation of those other inflammasomes remains to be investigated.

Pyroptosis. Current evidence suggests a crucial role of pyroptosis in liver diseases of all aetiologies[47]. Hepatocytes undergo pyroptosis in the context of NASH and liver fibrosis; elevated expression levels of inflammasome components, including NLRP3, pro-IL-18, pro-IL-1β and caspase 1 have been detected in individuals with NASH as well as in experimental mouse models of NASH[48–50]. Caspase 1 activity is elevated in the liver and serum of patients with NASH and fibrosis ($n = 63$), correlated with the severity of the disease and the presence of fibrosis[50]. In hepatocytes, pyroptosis is often triggered by the activation of Toll-like receptors by various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and consequent inflammatory cytokines, resulting in activation of inflammasomes such as NLRP3 (ref. 51). Once activated, inflammasomes can activate caspase 1, which enables the release of inflammatory cytokines such as IL-1β and IL-18, and induce pyroptosis by causing the formation of membrane pores, leading to cell swelling and rupture[51].

Moreover, Gaul and colleagues demonstrated that the activation of HSCs and subsequent spontaneous liver fibrosis in mice occurred with hepatocyte-specific NLRP3 hyperactivation, which triggered caspase-1-dependent pyroptosis in hepatocytes[50]. Also, in this study, in vitro investigations on primary mouse and human hepatocytes demonstrated the release of NLRP3 protein particles by pyroptotic hepatocytes, which were internalized by HSCs leading to their activation, promoting the production of extracellular matrix and fibrosis[50]. Additionally, increased levels of the pyroptosis-inducing protein gasdermin D (GSDMD) have been observed in the liver of patients with NAFLD ($n = 15$) or NASH ($n = 14$) compared with the levels in healthy liver tissue ($n = 16$), particularly in those with advanced fibrosis[52]. Notably, the cleaved GSDMD N-terminal domain (GSDMD-N) correlated positively with liver fibrosis and the NAFLD activity score of patients[52]. In the same study, the global knockout of GSDMD reduced liver inflammation and fibrosis in MCD-fed mice, whereas hepatocyte-specific overexpression of GSDMD-N caused liver damage and worsened inflammation[52].

ROS production. In the context of NAFLD and NASH, it is noteworthy that ROS not only contribute to oxidative stress but can also induce ER stress by oxidizing resident proteins[53]. Hence, understanding the interrelationship between ER stress and oxidative stress in hepatocytes is crucial for elucidating the underlying mechanisms that drive the development of NASH. The increased oxidation of FFAs during NAFLD and mitochondrial dysfunction during NASH lead to incomplete fatty acid oxidation (FAO) and the generation of reactive ROS in the liver[54,55]. FFAs are then handled by parallel pathways such as peroxisomal β-oxidation and microsomal ω-oxidation, leading to more ROS production[56]. ER stress and impaired protein folding can also lead to ROS generation[57,58]. Although the major source of ROS in the cell is mitochondria[59], ER physiological protein folding produces up to 25% of cell ROS[60,61]. An accumulation of ROS can trigger the oxidation of ER-resident proteins, leading to ER stress and causing a vicious cycle[57,62].

In mouse liver, through the phosphorylation of the nuclear factor erythroid-derived 2-like 2 (NFE2L2; also known as NRF2), the PERK branch of the UPR induces the transcription of several important antioxidant enzymes, which mitigates the damaging effects of excessive levels of ROS[17,63]. In NASH, the NRF2 pathway might protect against oxidative stress-induced inflammation. Mice fed an MCD and deficient in *Nrf2* exhibited a worsening of NASH features owing to elevated oxidative stress and the production of pro-inflammatory cytokines in the liver[64,65]. Conversely, prolonged pharmacological activation of *Nrf2* suppressed oxidative stress and induced the release of inflammatory cytokines and profibrotic factors[65]. In mouse models of NAFLD and in in vitro models, pharmacological *Nrf2* activation was associated with lower lipid accumulation, oxidative stress and inflammation in the liver[66,67]. In rats fed a choline-deficient L-amino acid-defined (CDAA) diet, pharmacological stimulation of *Nrf2* resulted in fibrosis attenuation[68]. Similarly, mice with genetic activation of *Nrf2* through knockdown of KEAP1, a protein that inhibits *Nrf2* transcriptional activity under physiological conditions, were less susceptible to developing NASH owing to the MCD[69]. These investigations demonstrate that NRF2 limits NASH progression by activating antioxidative stress response genes and modulating hepatocyte fatty acid metabolism.

It is important to note that, although animal studies provide valuable mechanistic insights, further research is needed to validate these findings in patients with NASH and explore the translational potential of targeting ER stress pathways.

Mitochondrial dysfunction

Mitochondrial dysfunction is a hallmark feature of NASH[55,70–72]. In the context of NASH, elevated levels of FFAs in the liver have a critical role in initiating mitochondrial dysfunction[55]. Impaired mitochondrial function can cause cellular energy metabolism disruption and oxidative stress, leading to a cascade of pathological events[55]. This section discusses the relationship between mitochondria, FFAs and the development of NASH, elucidating the fundamental mechanisms involved.

Mitochondria, the powerhouses of the cell, are responsible for energy production through the oxidation of substrates such as fatty acids, pyruvate and amino acids. This process, called oxidative phosphorylation, tightly couples the oxidation of such substrates with ATP synthesis, and is regulated by many intracellular and extracellular factors[73,74]. During fasting, FFAs released by the adipose tissue are β-oxidized by the mitochondria in the liver[75,76]. In a fed state, high serum glucose and insulin levels stimulate de novo lipogenesis[59,77], leading to the synthesis of FFAs that are stored as triglycerides in the liver or secreted as VLDLs. FAO involves the transfer of NADH and $FADH_2$ to the mitochondrial respiratory chain (MRC), which supplies protons and electrons to generate ATP. Mitochondria are the major source of ROS production and have a crucial role in redox balance and energy metabolism[78–80]. Most electrons are transferred to the last complex of the MRC, the cytochrome *c* oxidase, where they interact with protons and oxygen to generate water. However, some can react directly with oxygen to form the superoxide radical anion, particularly via complexes I and III or the MRC and some enzymes of the FAO pathway[81,82].

Maintaining an appropriate level of mitochondrial ROS is crucial as, under physiological conditions, ROS activate multiple intracellular and extracellular signal transduction pathways[83]. However, excessive ROS can overwhelm antioxidant defences, and oxidative stress occurs[84]. Oxidative stress can cause cellular dysfunction and pathological processes as ROS can damage proteins, lipids (lipid peroxidation) and nucleic acids, ultimately resulting in cell death[73]. Low-intensity ROS production is required for metabolic flexibility, and moderate

ROS release might regulate inflammatory mediators — high levels of ROS initiate processes such as apoptosis and autophagy[85–87].

Mitochondrial adaptation and dysfunction in NAFLD and NASH

Mitochondria oxidative capacity varies substantially in obesity and NAFLD, primarily because of body mass, age, insulin sensitivity and type 2 diabetes, chronic alcohol consumption and genetics. Under normal circumstances, the liver maintains a balance between lipid oxidation and lipogenesis. However, this balance is altered in NAFLD, leading to lipid accumulation and insufficient FFA oxidative degradation or secretion associated with mitochondrial adaptation[70]. In response to increased FFA flux, liver mitochondrial activity is increased. Studies have shown a fourfold or fivefold increase in mitochondrial O_2 consumption in individuals with obesity, with or without NAFLD, compared with that in individuals with normal weight and a healthy liver[54]. Similarly, increased mitochondrial function was observed in diet-induced mouse and rat models of liver steatosis and metabolic syndrome[88,89]. Progressive NAFLD is associated with ultrastructural mitochondrial abnormalities, such as increased size, loss of mitochondrial cristae, paracrystalline inclusions or linear crystalline inclusions in swollen mitochondria[87,88]. The MRC might become uncoupled, and its activity might decrease, inducing hepatic depletion of ATP[72,90,91]. These mitochondrial changes are triggered by modifications in mitochondrial ROS production and signalling pathways, mitochondrial biogenesis, mitophagy, increased liver FFAs, lipid peroxidation products and secretion of tumour necrosis factor (TNF). Although early-stage NAFLD is associated with increased levels of mitochondrial respiration compared with that in healthy individuals, NASH is characterized by mitochondrial dysfunction[54]. Despite higher mitochondrial biogenesis, liver tissue from patients with NASH exhibits 31% to 40% lower maximal respiration, mitochondrial uncoupling and increased proton leak[54]. This dysfunction is accompanied by higher ROS production, lipid peroxidation, oxidative DNA damage associated with depletion of antioxidant defence and higher inflammatory response[54]. Similarly, animal models of NASH with and without fibrosis demonstrate impaired mitochondrial function[42,43]. For example, NASH mice fed a HFD had decreased mitochondria antioxidant levels, increased mitochondrial ROS production and impaired mitochondrial function associated with higher liver TNF protein levels[92]. Similarly, mice fed a CDAA–HFD with NASH and fibrosis had higher hepatic oxidative stress and lipid peroxidation, and reduced activity of MRC complex I and ATP production[93]. These results indicate that the initial mitochondrial adaptations seen in the early stages of NAFLD are lost in NASH, leading to oxidative stress and inflammation.

Dysfunctional mitochondria generate excessive ROS, which leads to abnormal redox signalling, hepatocyte DNA damage, apoptosis or necrosis, immune cell infiltration and HSC activation, further contributing to the transition from NAFLD to NASH and fibrosis, as has been shown in mouse models[94]. FFA overload increases mitochondrial FAO to limit hepatic fat deposition and lipotoxicity but increases ROS generation and disrupts redox homeostasis[95,96]. In isolated mitochondria from bovine heart, FFAs inhibit MRC complexes I and III, blocking electron transport and increasing ROS production[97,98]. Excessive ROS can further impair mitochondrial function, generate additional ROS[99] and trigger mitochondrial permeability transition pore opening, releasing cytochrome *c* and leading to apoptosis as was shown in vivo[78,99]. Also, mitochondrial ROS can oxidize unsaturated FFAs, inducing lipid peroxidation. In MCD-fed rats, steatohepatitis development was associated with the presence of end products of lipid peroxidation and mitochondrial injury[100]. These highly reactive products, such as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE), can damage mitochondrial DNA (mtDNA) and react with MRC proteins[54,101–103]. Although adaptive changes such as mitochondrial biogenesis can occur, these effects can partially impair electron transmission in the MCR and increase ROS generation[59].

Accumulation of damaged mitochondria due to alterations in the mitophagy pathway can lead to cell necrosis and the release of danger signals that activate the expression of inflammatory cytokines, promoting the development of chronic liver inflammation[59]. Similar to bacterial PAMPs that activate an inflammatory response, hepatocyte injury releases mitochondrial DAMPs such as mtDNA and mitochondrial formyl peptides[59,104]. These mitochondrial DAMPs can trigger innate immune responses and the production of inflammatory cytokines to promote liver inflammation[104]. Mitochondrial DAMPs were elevated in the circulation of individuals with NAFLD and NASH[105]. Serum mtDNA levels were, on average, sevenfold higher in patients with NAFLD at various stages of the disease, with levels reaching up to 17-fold above normal. Patients with active NASH (NAS 4–8, $n = 90$, versus minimal disease activity NAS 0–3, $n = 24$) and especially those with strong histological indications of fibrosis (stage F2–4, $n = 46$) had higher circulating mtDNA levels than those with little or no fibrosis (stage F0–1, $n = 68$)[105].

CYP2E1 activity

CYP2E1 has also emerged as a potential source of excessive ROS production. In addition to the ER, CYP2E1 was shown in mice to localize to the mitochondria[106,107]. In the liver, it metabolizes substances such as ethanol, FFAs and carbon tetrachloride (CCl_4). During its catalytic cycle, CYP2E1 generates ROS and harmful metabolites from xenobiotics[108,109]. CCl_4 radicals rapidly promote lipid peroxidation[110]. CYP2E1 was induced in mice and humans with NAFLD, and alcohol-related liver disease (ALD)[109,111–115]. Currently, the mechanism by which CYP2E1 promotes NAFLD progression is unknown, although CYP2E1-mediated ROS production might have a substantial role[111,116]. Mice fed a HFD for 10 weeks developed NASH associated with lipid peroxidation and increased CYP2E1 expression and activity, whereas wild-type and *Cyp2e1*-null mice, also fed a HFD, only developed simple steatosis[117]. Mice fed a cholesterol-containing fast-food diet developed NASH and fibrosis accompanied by nitroxidative and ER stress, but *Cyp2e1*-null mice, also fed a fast-food diet, did not[118,119]. Conversely, in rats, the addition of fructose to drinking water led to the induction of CYP2E1 activity and liver steatosis, inflammation and fibrosis[119].

Furthermore, in the liver of patients with NASH, CYP2E1 was upregulated compared with the levels in patients with simple steatosis and controls, and was associated with higher protein oxidation and lower antioxidant defence[120]. As liver mitochondria also contain CYP2E1, CYP2E1 upregulation might be linked to mitochondrial dysfunction[107]. Indeed, CYP2E1-mediated oxidative stress and lipid peroxidation can alter mitochondrial constituents, including the MRC, as has been shown in vitro[121,122]. This MRC alteration can lead to excessive electron leaking in MRC complexes I and III, which enhances ROS production[123,124]. Hence, CYP2E1-mediated ROS overproduction, decreased ROS detoxication, and lipid peroxidation-derived reactive aldehydes might modify mitochondrial function.

Oxidative stress and downstream signalling pathways

Several studies have highlighted downstream signalling pathways induced by cellular stress. For example, during the ROS-induced process of lipid peroxidation, aldehyde products such as 4-HNE can activate nuclear factor-κB (NF-κB), which induces the expression of

pro-inflammatory genes, including cytokines and chemokines, and participates in inflammasome regulation[125,126]. General oxidative stress can also induce NF-κB[127].

Mitochondrial ROS can activate mitogen-activated protein kinases (MAPKs), including JNK[87,128], and the energy sensor AMP-activated protein kinase (AMPK). In the MAPK pathway, chronic oxidative stress, secreted TNF or ER stress activates apoptosis signal-regulating kinase 1 (ASK1; also known as MAP3K5), leading to p38–JNK pathway activation, hepatocyte apoptosis and fibrosis[87]. JNK is activated in the liver of patients with NASH, and genetic inactivation of JNK isoforms in experimental animal models reduces hepatic steatosis[31,129]. Prolonged JNK activation induced by ROS contributes to cell death via the inactivation of the MAPK phosphatases in vitro[130]. Phosphorylated JNK translocates into the mitochondria to inhibit the MRC, increasing ROS production[87,131,132]. ROS subsequently activate ASK1 and MKK4, which keep JNK active[62]. In mice, blocking phosphorylated JNK binding to mitochondria reduced mitochondrial ROS generation and prevented liver damage[133].

In response to cellular energy depletion, AMPK is activated, promoting energy-producing metabolic pathways (FAO) and inhibiting energy-consuming pathways (lipogenesis, gluconeogenesis)[128]. The generation of H_2O_2 by mitochondria also activates AMPK, which promotes NRF2 to upregulate the expression of antioxidant enzymes in murine models[66,67,86]. In the liver of *Nrf2*-deficient HFD-fed mice, lower levels of active phosphorylated AMPK were associated with greater induction of lipogenic genes, decreased expression of FAO genes and increased fatty acid synthesis[86]. Compared with $Nrf2^{+/+}$ primary hepatocytes, $Nrf2^{-/-}$ hepatocytes showed decreased glucose and FAO, that was partially recovered by treatment with AMPK activators[86]. Although some molecular mechanisms for AMPK-mediated *Nrf2* activation have been proposed, the exact mechanisms in NAFLD and NASH remain to be elucidated. Thus, therapeutic targeting of AMPK in patients with NASH represents an exciting strategy to promote FAO and glycolysis[59].

Excessive ROS production impairs mitochondrial dynamics, leading to mitophagy arrest and, therefore, inducing the mitochondrial UPR to promote cell recovery and preservation of the mitochondrial network, as shown in mice and in vitro[134]. Mitophagy, the removal of dysfunctional mitochondria, is protective against the onset of NAFLD, as shown in mice[135]. It reduces oxidative stress and preserves mitochondrial bioenergetics to regulate liver metabolism and avoid cell death[76].

In addition to the oxidative stress pathways discussed above, another emerging mechanism involved in NASH pathogenesis is ferroptosis, a distinct form of cell death characterized by iron-mediated oxidative stress and lipid peroxidation in hepatocytes[136–138]. Oxidative stress induced by high levels of ROS and lipid peroxidation in hepatocytes resulting from the iron-mediated Fenton reaction and enzymatic oxygenation can lead to ferroptosis[139]. In mouse models of steatohepatitis, ferroptosis was associated with the onset of inflammation during the early stages of NASH[136]. In a choline-deficient methionine-supplemented mouse model of NASH, inhibition of ferroptosis protected hepatocytes from necrotic cell death and suppressed immune cell infiltration and inflammation[136]. These findings suggest that hepatic ferroptosis has a critical role as a trigger for initiating inflammation in NASH. It also indicates that targeting ferroptosis could be a potential therapeutic approach for preventing the onset of NASH.

Stress mechanisms in hepatocytes and fibrogenesis independent of NASH

Although our previous discussion is primarily focused on the cellular stress mechanisms within hepatocytes in the context of NASH, it is important to note that current studies have demonstrated that cellular stress pathways in hepatocytes also contribute to fibrogenesis independent of NASH. Therefore, to comprehensively understand the pathogenesis of liver fibrosis, we must explore the distinct cellular stress mechanisms that drive fibrotic progression. This section summarizes studies implicating cellular stress pathways in liver fibrosis, highlighting the relationship between hepatocyte stress and fibrotic remodelling.

Endoplasmic reticulum stress

In addition to NAFLD and NASH, ER stress is observed in various liver diseases, including ALD and chronic viral hepatitis. Patients with chronic liver disease and severe fibrosis have higher levels of hepatic ER stress markers than those with milder fibrosis[140]. For example, PERK phosphorylation levels were increased in severe fibrosis compared with mild fibrosis[140]. Similarly, BiP and CHOP proteins were increased in patients with chronic HCV infection with severe fibrosis compared with the levels in those with mild fibrosis (METAVIR scores, 0–1 versus 2–4)[140].

Multiple studies in mouse models of fibrosis have demonstrated the involvement of ER stress in hepatocytes and that its inhibition decreases fibrosis development[141–143]. For example, in a CCl_4-induced rat model of fibrosis, resveratrol treatment alleviated fibrosis and decreased expression of the ER stress proteins CHOP and BiP and apoptosis-related proteins[141]. In a bile duct ligation (BDL)-induced mouse model of fibrosis, investigations in mutated α_1-antitrypsin Z protein mice revealed that *Chop* and *Grp78* mRNA upregulation was associated with increased sensitivity to hepatocyte death and fibrosis. Although the mechanism is unclear, this study suggests that ER stress induces cholestasis-induced hepatocyte injury[142]. Other studies in mice showed that after BDL-induced cholestasis, *Chop* and *Bax* mRNA expression levels were induced in wild-type mice, and *Chop*-deficient mice showed attenuated fibrosis. In vitro experiments further showed that the accumulation of bile acids led to hepatocyte necrosis and apoptosis, which were inhibited in isolated hepatocytes from *Chop*-deficient mouse, providing a potential explanation for the attenuation of liver fibrosis[143]. CHOP-mediated apoptosis of hepatocytes might be a primary mechanism linking ER stress and fibrosis in the liver.

In CCl_4-induced mouse models of fibrosis, it was shown that STING, an ER mitochondria-associated membrane protein, promoted hepatocyte apoptosis and liver fibrosis[144]. These findings also suggest that innate immune signalling regulates hepatic fibrosis through hepatocyte death. Notably, the expression of STING in hepatocytes remains debatable[145,146], and the precise role of STING in chronic liver injury, particularly NASH, seems to be dependent on the timing and stimulus[147].

Oxidative stress and mitochondrial dysfunction

As in NAFLD and NASH, accumulating evidence indicates that oxidative stress and mitochondrial dysfunction are involved in the physiopathology of liver fibrogenesis. In human liver biopsy samples, patients with advanced fibrosis associated with HBV infection ($n = 46$) or HCV infection ($n = 6$) exhibited alterations in mitochondrial function and homeostasis compared with patients with chronic liver disease and less advanced fibrosis ($n = 121$ and $n = 17$, respectively)[148,149]. Proteome analysis of biopsy samples from patients with later stages of HCV-induced fibrosis revealed the downregulation of mitochondrial proteins compared with early-stage fibrosis biopsy samples, suggesting an impairment of mitochondrial processes and oxidative stress response[148]. A study published in 2022 in patients with NASH, or HBV-associated or HCV-associated fibrosis showed that patients with advanced

fibrosis (stage F3–4) had mtDNA oxidative damage, alterations of mitochondrial function, mitochondrial UPR, biogenesis, mitophagy and liver inflammation when compared with patients with no or mild–moderate fibrosis (stage F0–2)[149]. In addition, in vitro studies in HepG2 cells that showed HBV replication or transient expression of the HBV X protein led to the generation of ROS and reactive nitrogen species, mtDNA damage and impairment of the MRC complexes; these effects were partly inhibited by a ROS scavenger or an inducible nitric oxide synthase (iNOS) inhibitor[149].

Several in vivo experimental studies have demonstrated the presence of oxidative stress and mitochondrial dysfunction in experimental models of fibrosis. CCl_4-induced mouse and rat models of fibrosis showed decreased activity of MRC complexes as well as decreased ATP levels, ROS production, lipid peroxidation and mtDNA oxidative damage in the liver[150–153]. Similarly, in the liver of mice with high-dose radiation-induced liver fibrosis, highly irradiated fibrotic areas exhibited markers of ROS and antioxidant depletion along with lipid peroxidation and mtDNA damage. Additionally, isolated hepatocytes displayed impaired mitochondrial respiration in vitro[154]. Inhibition in ROS generation during the early stages of fibrosis induction has been shown to attenuate fibrosis. For example, in chronic CCl_4-injected mice, overexpression of the mitochondrial anti-apoptotic factor Bcl2, specifically in hepatocytes, resulted in decreased mitochondrial dysfunction, ROS production, mtDNA oxidative damage and protection against early fibrogenesis. However, this protection was insufficient to prevent fibrosis progression to advanced stages[151]. Comparably, administration of the mitochondria-targeted antioxidant mitoquinone mesylate (MitoQ) compound in the CCl_4-induced mouse model decreased lipid peroxidation, cell death and inflammation. This was accompanied by attenuated liver fibrosis and collagen deposition and in vitro inhibition of activation of isolated HSCs[150]. These findings emphasize the importance of mitochondrial oxidative stress, mtDNA damage and related abnormalities in mitochondrial function and dynamics in liver fibrosis.

In the context of liver fibrosis, mitochondrial dynamic balance is impaired. In a CCl_4-induced mouse model of fibrosis, mitochondrial fragmentation increased, leading to cell death and HSC activation. Mitochondrial fragmentation positively correlated with the liver fibrosis stage, and pharmacological suppression of mitochondrial fission decreased cell death, HSC activation and liver fibrosis[155]. According to the proposed mechanism, damaged mitochondria trigger RIPK1–MLKL-dependent hepatocyte death, which enhances the activation of HSCs and exacerbates liver fibrosis[155]. Similarly, in mice with CCl_4-induced fibrosis, oxidative stress resulted in mtDNA damage, leading to abnormal mitochondrial fission associated with hepatocyte epithelial–mesenchymal transition (EMT) in liver tissue and increased liver fibrosis[156]. Interestingly, antioxidants apocynin and mito-TEMPO effectively reduced oxidative stress-induced liver fibrosis[156]. Furthermore, inhibition of mitochondrial fission with Mdivi-1 alleviated histopathological changes in the fibrotic liver by decreasing collagen I and αSMA expression. Restoring oxidative stress-impaired mitochondrial biogenesis was shown to mitigate hepatocyte EMT, indicating a protective role of PGC1α, the major regulator of mitochondrial biogenesis[157], in preventing oxidative stress-induced liver fibrosis. This finding was supported by observations of more severe liver fibrosis in *Pgc1a*-knockout mice[156].

Concerning mitochondrial DAMPs, one study found in a thioacetamide-induced mouse model of liver injury that a fibrosis-susceptible mouse strain had impaired macrophage-mediated clearance of dead hepatocytes. This process led to the release of mitochondrial DAMPs and activation of HSCs adjacent to areas of incomplete efferocytosis[105]. These results suggest that efficient phagocytic clearance of dead hepatocytes by macrophages is essential for scarless recovery and that mitochondrial DAMPs have a central role in liver fibrosis[137]. Targeting these pathways could be pertinent for the development of antifibrotic therapies.

CYP2E1 induction and activity

CYP2E1 has been implicated in the development of liver fibrosis in various mouse models and patients owing to its role in inducing oxidative stress, ER stress and inflammation[118,119,158,159]. Interestingly, in mice genetically modified to express human *CYP2E1* only, alcohol feeding induced greater inflammation and fibrosis associated with oxidative stress and lipid peroxidation than in wild-type mice. Mechanistically, mouse livers expressing human *CYP2E1* exhibited higher transcriptional activity of NRF2 with greater induction of detoxifying enzyme expression compared with livers from wild-type mice[158]. However, the specific function of CYP2E1 in the development of liver fibrosis has been the subject of minimal research to date, and the underlying mechanisms deserve additional investigation.

In conclusion, various cellular stress mechanisms in hepatocytes have a role in hepatic fibrogenesis, in the context of both NASH and other types of chronic liver disease. Inhibition of ER stress has shown encouraging outcomes in fibrosis attenuation. Mitochondrial dysfunction, oxidative damage, mitochondrial dynamics impairment and CYP2E1 activity also influence the fibrotic process. Understanding these various cellular stress pathways offers substantial knowledge regarding the development of liver fibrosis and emphasizes potential therapeutic targets for antifibrotic therapies. Additional investigation is required to elucidate the complex interactions between those stress mechanisms and their specific role in fibrogenesis.

Stress pathways in HSCs and fibrosis pathogenesis

Interestingly, current studies have implicated cellular stress pathways in HSCs, the primary cell type responsible for extracellular matrix deposition. In this section, we discuss the studies implicating the roles of stress pathways in HSC activation and fibrosis progression. These pathways are illustrated in Fig. 3.

Endoplasmic reticulum stress and HSC activation

Prior studies in mice have demonstrated a correlation between high expression levels of ER stress-related genes and HSC activation[140,160]. For example, increased gene expression of *Atf4* (PERK downstream gene), *Herpud1* (a marker of *Atf6* activation) and *sXbp1* were observed within 2 h of in vitro culture activation of mouse HSCs[160]. Consistently, rat HSCs that underwent spontaneous in vitro culture activation showed a dramatic increase in cell ER population and ER stress proteins and mRNA levels, such as PERK, CHOP and BiP[140]. The UPR was sustained in HSCs of mice that received up to eight CCl_4 injections, suggesting that chronic injury maintains high levels of the UPR in HSCs[160]. Similar findings were observed in mice fed a HFD for 20 weeks[140]. Administration of tunicamycin to trigger ER stress increased expression of fibrosis marker genes such as *Tgfb1* and *Acta2* in rat HSCs[140]. Furthermore, to examine the effect of ER stress in HSCs on the development of liver fibrosis, Koo and colleagues transfected mice with a lentiviral construct comprising the gene encoding BiP — a well-characterized ER chaperone protein that regulates the UPR, downstream from the αSMA promoter. Overexpression of BiP reduced ER stress in HSCs, decreased collagen accumulation in the CCl_4-mouse liver, and prevented the ability of tunicamycin to

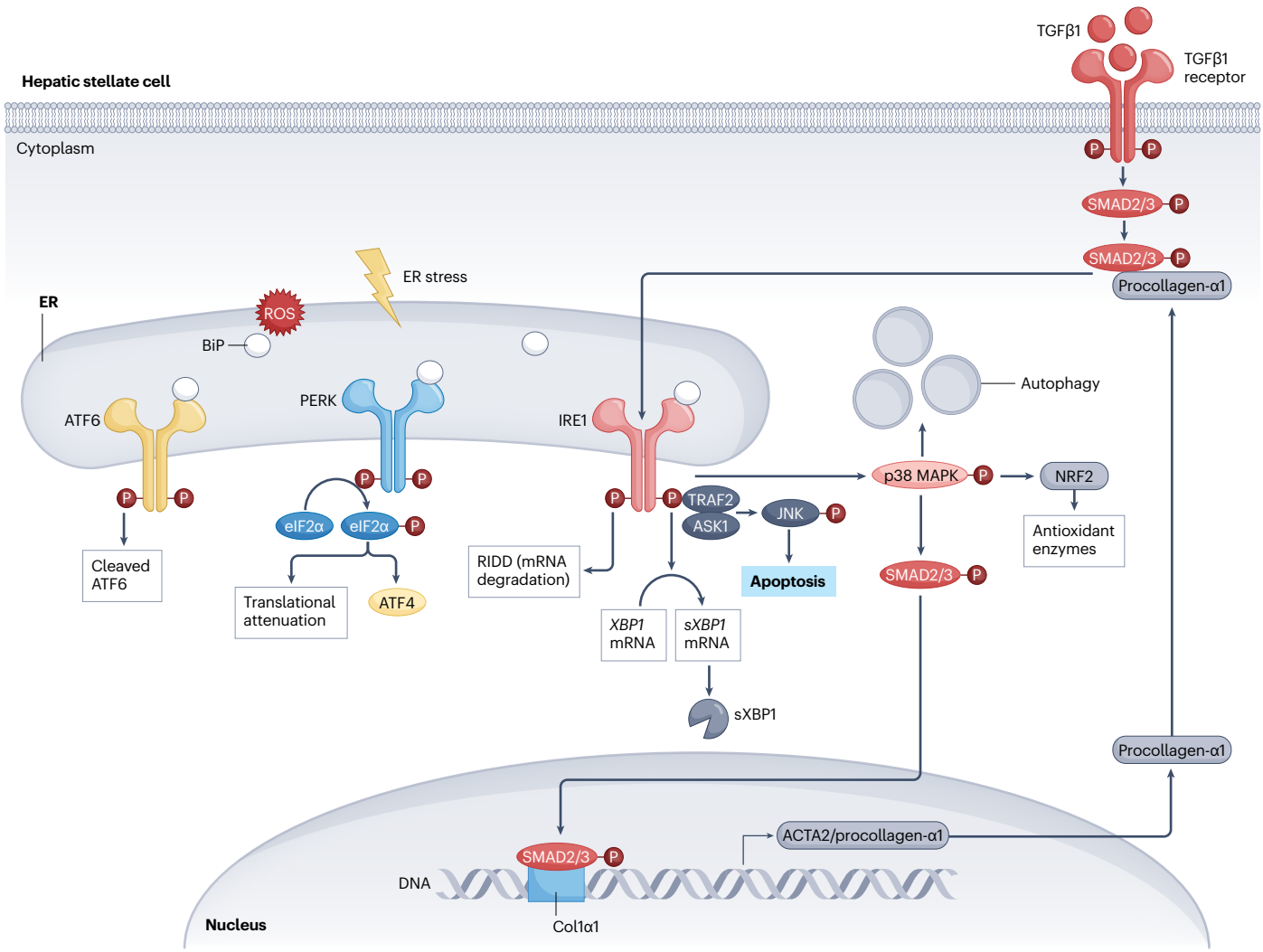

Fig. 3 | Overview of cellular stress in hepatic stellate cells. The profibrotic transforming growth factor-β1 (TGFβ1) cytokine induces inositol-requiring enzyme 1 (IRE1) phosphorylation in a SMAD2/3–procollagen-α1-dependent manner, leading to p38 phosphorylation and activation of hepatic stellate cells (HSCs). Activation of the unfolded protein response has been associated with the induction of autophagy, which promotes the activation of HSCs. Additional studies demonstrated that oxidative stress and mitochondrial damage promote HSC activation and spontaneous liver fibrosis by activating the NOD-, LRR- and pyrin domain-containing protein 3 inflammasome complex. ASK1, apoptosis signal-regulating kinase 1; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; Col1α1, collagen type I-α1; ER, endoplasmic reticulum; JNK, JUN amino-terminal kinase; NRF2, nuclear factor erythroid 2-related factor 2; p38 MAPK, p38 mitogen-activated protein kinase; PERK, protein kinase R-like endoplasmic reticulum kinase; RIDD, regulated IRE1-dependent decay; ROS, reactive oxygen species; TRAF2, TNF receptor-associated factor 2; XBP1, X-box binding protein 1.

induce profibrotic gene expression[140]. Consistently, pharmacological inhibition of the UPR decreased HSC activation in mice and rats[140,161]. Taken together, these studies suggest that ER stress accompanies HSC activation, and induction of ER stress in HSCs promotes liver fibrosis development.

Regulators of UPR signalling pathway in HSCs

Studies have aimed to identify regulators of UPR signalling in HSCs. Interestingly, Mannaerts and colleagues observed that the UPR was insufficient to drive HSC activation in 3D-spheroid cultures, suggesting that other stimuli might be required. The researchers found that the UPR response was a JNK1-dependent early event in HSC activation and was modulated by nonsense-mediated decay[160]. Others have highlighted the critical role of TGFβ in inducing the UPR and promoting HSC activation in mice and humans. For example, Liu and colleagues found that TGFβ induced the UPR via IRE1 in vitro, which led to a feedforward signalling pathway via the UPR-associated transcription factor CCAAT/enhancer binding protein β (C/EBPβ) to promote fibrogenesis[162]. The investigators demonstrated that TGFβ induced IRE1 phosphorylation in a SMAD2/3 procollagen-α1 (procollagen type I)-dependent manner. Furthermore, ASK1 and JNK signalling promoted phosphorylation of C/EBPβ, which is crucial for TGFβ or IRE1-mediated

HSC activation and hepatic fibrogenesis. The researchers also found that the transcriptional regulator p300 was critical for TGFβ and UPR-mediated HSC activation and might act through a mechanism involving C/EBPβ[162].

Other in vitro studies have demonstrated a link between UPR and cellular autophagy in promoting HSC activation. Autophagy is an important driver of HSC activation and fibrosis[163]. Rapid upregulation of autophagy in HSCs is an adaptive response under cellular stress conditions. Autophagy provides energy to support HSC activation through lipid droplet mobilization, FFA liberation and mitochondrial β-oxidation[164]. Blockade of the IRE1 pathway reduced autophagy and decreased HSC activation in mice and humans in a p38 MAPK-dependent manner[164]. This study supports the relationship between induction of autophagy and p38 MAPK under ER stress conditions and provides a rationale to explore the specific blockade of IRE1 in HSCs as an antifibrotic strategy. In addition, XBP1 overexpression induced COL1A1 expression in primary mouse HSCs, which were diminished by depletion of the autophagy mediator ATG7. Interestingly, this XBP1-mediated UPR-stimulated autophagy and collagen production is independent of TGFβ signalling[162,165]. This finding indicates that targeting the XBP1 branch of the UPR might be an alternative approach to targeting the TGFβ pathway.

Targets of endoplasmic reticulum stress in HSCs

Studies have aimed to elucidate downstream targets of ER stress in HSC. For example, a study in mice and humans observed that ER stress activated phosphorylated PERK, which led to a decrease in heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and microRNA 18a (MIR18A), a suppressor of SMAD2 expression[140]. This dysregulation led to TGFβ–SMAD2 pathway activation, which promoted HSC activation. Consistently, patients ($n = 44$) with severe fibrosis had increased levels of phosphorylated PERK and reduced levels of HNRNPA1 in HSCs compared with patients with mild fibrosis[140]. This study suggests that the PERK–HNRNPA1–MIR18A–SMAD2 signalling axis might consists of druggable targets to ameliorate fibrosis[140].

An in vitro study by Ruiz de Galarreta and colleagues showed that brefeldin A (BFA), an ER stress inducer, triggered an IRE1-p38 MAPK–SMAD axis that promoted increased expression levels of collagen type 1 in HSCs[166]. Another study in mice highlighted the involvement of IRE1α endonuclease activity (RNase activity) in microRNA processing[161]. IRE1 facilitated HSC activation by inactivating miR-150, a repressor of MYB and αSMA expression in mouse liver[161]. Inhibition of IRE1α with 4μ8C attenuated TGFβ and BFA-induced activation of HSCs in vitro and reduced fibrosis in alcohol-induced and CCl_4-induced mouse models[161].

Maiers and colleagues uncovered Transport and Golgi organization 1 (TANGO1) protein as an important target of the UPR in a human HSC cell line[167]. The study showed that UPR signalling upregulates TANGO1 in response to TGFβ, which is mediated by the transcription factor XBP1. Depletion of TANGO1 protein suppressed collagen I secretion and promoted UPR-mediated apoptosis in HSCs. *Tango1*$^{+/-}$ mice experience decreased fibrosis in the CCl_4-induced and BDL-induced models. Mouse and human cirrhotic liver tissue demonstrated increased *TANGO1* mRNA levels[167]. The findings of this study taken together implicate the unique role of TANGO1 and the UPR in promoting collagen I secretion and fibrosis.

Endoplasmic reticulum stress and HSC apoptosis

Prior in vitro and in vivo studies have suggested promoting ER stress-mediated apoptosis in HSCs as a potential therapeutic strategy for treating fibrosis. For example, Lim and colleagues demonstrated that cannabidiol (CBD), the non-psychoactive component of the plant *Cannabis sativa*, elicited an ER stress response in a human HSC cell line that was characterized by activation of signalling cascades mediated by PERK, ATF6 and IRE1 (ref. 168). CBD promoted apoptosis by activating the IRE1–ASK1–JNK pathway[150]. Similar findings have been reported with caffeine treatment in vitro[169]. Another study in mice investigated the effect of deletion of heat shock protein 47 (HSP47), a collagen-specific molecular chaperone essential for the maturation and secretion of collagen. This study demonstrated that apoptosis is induced through ER stress by the deletion of HSP47 and inhibition of autophagy[170].

Oxidative stress and HSC activation

The contribution of glycolysis, glutaminolysis and mitochondrial oxidative phosphorylation has been explored in rat and human HSC activation. Smith-Cortinez and colleagues observed that glycolysis and mitochondrial respiration were increased in activated rat HSCs compared with quiescent HSCs, and were associated with mitochondrial fusion[171]. Human HSC proliferation and activation were suppressed by inhibiting glycolysis, glutaminolysis or mitochondrial oxidative phosphorylation[171]. Bae and colleagues similarly observed that activated HSCs had higher basal respiration, maximal respiration, ATP production, spare respiratory capacity and proton leak than quiescent HSCs in mice[172]. In additional studies, Bae, Chen and colleagues observed that astaxanthin and saikosaponin D inhibited oxidative phosphorylation, promoting HSC apoptosis and reducing HSC activation in vitro[172,173]. A study evaluated the role of mitochondrial uncouplers and found that they inhibit HSC activation by reducing ATP and ROS levels in vitro[174].

Studies have demonstrated that ROS participate in HSC activation; for example, Greenwel and colleagues found that the collagen type I promoter is regulated by ROS generation in HSC cells[175]. Key regulators of ROS have been identified in HSCs, including NOX. For example, in a study in mice, HSCs deficient in *Nox1* and *Nox2* had decreased ROS generation and failed to upregulate collagen type 1 and TGFβ in response to angiotensin II[176]. ROS generation was decreased in *Nox1*-knockout and *Nox2*-knockout mice and associated with reduced fibrosis in CCl_4-induced and BDL-induced mouse models[177]. Similarly, deficiency of NOX4 attenuated liver injury, inflammation and fibrosis in the CCl_4-induced mouse model, which was reproduced by treatment with a NOX1–4 dual inhibitor GKT137831 (ref. 177). NOX1 and NOX4 protein levels were increased in patients with cirrhosis ($n = 10$) compared with the levels in healthy individuals ($n = 7$)[177]. Sancho and colleagues observed in mice that NOX4 is necessary for HSC activation and the maintenance of the myofibroblast phenotype[178]. Sasaki and colleagues investigated the role of NOX4 in a mouse model of early alcoholic liver injury. It was observed that the *Nox4* promoter was induced in HSCs by acetaldehyde, and NOX4 increased the mRNA stability of *Ccr2*–*Ccl2*. Their findings suggest that this enhanced stability promotes the recruitment of inflammatory cells[179].

Other studies have demonstrated the important role of cytoglobin (CYGB), a globin which acts as a scavenger of ROS, in fibrogenesis. *Cygb*-deficient mice show high sensitivity to fibrosis development, and protein overexpression of CYGB in HSCs attenuated thioacetamide-induced liver fibrosis in mice[180–183]. In additional studies in humans, Okina and colleagues observed that TGFβ–pSMAD2 suppresses *CYGB* expression and primes HSCs for H_2O_2-triggered hydroxyl radical production and 8-hydroxyguanosine (8-OHdG) generation. In patients with NASH, immunohistochemical staining of liver sections

revealed colocalization of 8-OHdG and αSMA in the stroma of patients with advanced liver disease[184]. Taken together, these findings suggest that CYGB might represent a target for antifibrotic approaches.

Current studies have identified an NLRP3–IL-18 axis in HSCs. Activation of NLRP3 in mouse HSCs resulted in increased HSC activation and spontaneous liver fibrosis, and this effect was attenuated in mice deficient in the IL-18 receptor[185,186]. These findings suggest a role of NLRP3 and IL-18 signalling in HSC activation and fibrogenesis.

Other studies identified p66SHC, the 66 kDa isoform of SHC, a redox enzyme that mediates mitochondrial ROS generation, as a key regulator of HSC activation. In rat HSCs, knockdown of p66SHC inhibited mitochondrial ROS production and NLRP3 inflammasome activation, which was associated with decreased HSC activation. Overexpression of p66SHC led to the opposite effects, which were attenuated by mito-TEMPO, a mitochondrial ROS scavenger[187]. Angiotensin-(1–7) has also been shown to modulate redox balance by opposing angiotensin II. Specifically, angiotensin II increased NOX4 protein expression and ROS production and inhibited GSH and NRF2–ARE, activating the NLRP3 inflammasome in rat HSCs; angiotensin-(1–7) opposed the effects of angiotensin II in HSCs[188]. In a BDL-induced rat model of fibrosis, angiotensin-(1–7) reduced H_2O_2 content, NOX4 protein levels, and the NLRP3 inflammasome to attenuate fibrosis[188].

Purinergic receptor P2X7 (P2X7R) has also been identified as a regulator of ER stress in HSCs. Jiang and colleagues observed that depletion of P2X7R reduced protein expression of NLRP3 and αSMA and decreased type I collagen secretion in human HSCs[189]. Additional modulators of oxidative stress in HSCs include α-lipoic acid and its reduced form dihydrolipoic acid (DHLA). It was observed that DHLA inhibited rat HSC activation and ROS generation, which could be mediated by MAPK and PI3K–AKT pathways[190]. Similarly, pyrroloquinoline quinone, a natural antioxidant, exerted potent ROS-scavenging and antifibrotic activity in a mouse model of liver fibrosis. Pyrroloquinoline quinone suppressed upregulation of the receptor for activated C kinase 1 in activated HSCs[191].

Autophagy and oxidative stress in HSCs

Several studies in mice have demonstrated the interplay between autophagy, oxidative stress and HSC activation. For example, Xie and colleagues observed that in mice, ethanol treatment of HSCs increased autophagy and oxidative stress and promoted HSC activation, which was reversed with the inhibition of autophagy. Long-term ethanol feeding of rats increased the UPR response in HSCs, which triggered autophagy and the NRF2-dependent antioxidant response in a p38-dependent manner[192].

Mitochondrial dynamics and HSC activation

Zhou and colleagues investigated the role of mitochondrial fission on HSC activation in a study published in 2022 (ref. 193). They observed that mitochondrial fission was enhanced in TGFβ1-activated rat HSCs and mouse and human fibrotic livers. Fission 1 homologue (FIS1) overexpression was associated with increased mitochondrial fission, leading to HSC activation, and inhibiting mitochondrial fission with the dynamin inhibitor Mdivi-1 promoted HSC inactivation and apoptosis in mice. Consistently, Mdivi-1 treatment reduced CCl_4-induced fibrosis and promoted HSC apoptosis in mice. Furthermore, the researchers observed that inhibition of oxidative stress in vitro using MitoQ or Tempol, a free radical scavenger, inhibited mitochondrial fission and oxidative phosphorylation, resulting in HSC apoptosis. The findings of this study suggest that oxidative stress contributes to enhanced mitochondrial fission, oxidative phosphorylation and HSC activation, promoting fibrogenesis[193]. However, an in vitro study by Su and colleagues found that mitochondrial fission reduced HSC activation, proliferation and collagen accumulation[194]. In this study, metformin induced mitochondrial fission through increasing phosphorylation of AMPK–DRP1 and inhibiting mitofusin 1 and mitofusin 2 expression. This inhibition resulted in a decreased fibrogenic response of HSCs. Additionally, metformin treatment increased mtDNA copy number and enhanced glycolysis to suppress the proliferation and activation of HSCs[194].

Regarding mitochondrial fusion, a study by Zhu and colleagues also demonstrated in mice that overexpression of mitofusin-2, a mitochondrial fusion protein, in HSCs inhibited TGFβ1–SMAD2/3 signalling and decreased expression of type I, type III and type IV collagen. Furthermore, the investigators observed that mitofusin-2 overexpression ameliorated CCl_4-induced fibrosis[195].

Augmenter of liver regeneration (ALR) is a mitochondrial protein that has been implicated in the regulation of mitochondrial dynamics of HSCs. Ai and colleagues observed that *Alr* knockdown in mice activated HSCs and accelerated migration, and this process was associated with elevated mitochondrial calcium and increased ATP synthesis[196]. By contrast, *Alr* transfection decelerated HSC migration, enhanced mitochondrial fission and reduced ATP synthesis[154]. These studies highlight the effect of mitochondrial morphology and energy metabolism in HSC activation and fibrosis progression. More studies are needed using HSC-specific deletion of mitochondrial fusion and fission genes such as *Fis1*, *Mfn1* and *Mfn2* to elucidate the effect further. In addition, given that mitochondria are highly dynamic, details such as time and nutrient energy are critical in experimental design and might account for some of the differences observed.

Mitophagy

Few studies have been published on the role of mitophagy in HSC activation and liver fibrosis. Wang and colleagues observed that fine particulate matter promoted HSC activation by inducing mitophagy via activating the PINK1–Parkin pathway in vitro[197]. However, Dou and colleagues observed that PINK1–Parkin-mediated mitophagy was associated with inhibition of HSC activation and fibrosis attenuation in mice[198].

Ding and colleagues evaluated the role of mitophagy in the context of fibrosis reversal and observed that enhanced mitophagy promoted apoptosis in HSCs and the reversal of hepatic fibrosis[199]. Inhibition of mitophagy by treatment with cyclosporine suppressed HSC apoptosis and increased hepatic fibrosis in mice. Additionally, the investigators observed that BCL-B, a member of the BCL-2 family, inhibited Parkin phosphorylation and apoptosis in human HSCs. The researchers concluded that BCL-B-dependent mitophagy regulated HSC apoptosis during fibrosis regression[199]. These studies illustrate that defective mitochondria and their clearance regulated the fibrogenic properties of HSCs and their fate in fibrosis regression.

Nitrosative stress in HSCs

Unlike oxidative stress, nitrosative stress through reactive nitrogen species such as nitric oxide inhibits HSC activation and proliferation, and collagen gene expression[200]. For example, polymeric nanoparticles decorated with vitamin A were used to deliver nitric oxide to HSCs in vivo. Released nitric oxide inhibited collagen I and αSMA expression in primary rat HSCs, and reduced portal hypertension in the liver[200]. Nitric oxide, a paracrine-acting soluble gas, is synthesized by NOS from

L-arginine[201]. Several NOS isoforms have been described, including neuronal NOS (nNOS, or NOS1), iNOS (or NOS2) and endothelial NOS (eNOS, or NOS3)[201]. In the thioacetamide-induced mouse model of fibrosis, pharmacological inhibition of iNOS upregulated procollagen-α1, MMP13, TIMP1 and PAI1 protein expression in the liver and promoted exacerbation of fibrosis; these findings suggest a substantial antifibrotic role of NOS2 (ref. 202). Furthermore, other studies have implicated iNOS–nitric oxide as a central mediator of adiponectin-induced fibrosis attenuation. Dong and colleagues observed in mice that adiponectin upregulated *Nos2* mRNA and NOS2 protein expression in HSCs and decreased HSC proliferation in a nitric oxide-dependent manner. Additional mechanistic experiments demonstrated that adiponectin-induced NOS2–nitric oxide activation is regulated through adiponectin receptor 2–AMPK–JNK–ERK1/2–NF-κB signalling[203].

Fibrogenesis and stress response in liver sinusoidal endothelial cells and macrophages

Compared with the literature on hepatocytes and HSCs, fewer studies have explored the role of cellular stress pathways in immune cells. In this section, we briefly discuss data involving LSECs, macrophages and fibrosis pathogenesis.

Liver sinusoidal endothelial cells

Endoplasmic reticulum stress. Although studies in endothelial cells from other organs have shown a role of ER stress in endothelial dysfunction[204,205], the effect of ER stress in LSECs and its contribution to fibrogenesis have been unexplored and require additional studies.

Oxidative stress. Studies have demonstrated the importance of capillarization on LSEC phenotype. Capillarization refers to a lack of fenestration and the development of a more continuous basal membrane[206–209]. In vitro studies with LSECs cocultured with HSCs demonstrated the role of capillarized LSECs on HSC activation; specifically, LSECs from normal rat liver prevented HSC activation and promoted reversion to quiescence, whereas capillarized LSECs did not achieve this phenotype[210]. The fenestrated LSEC phenotype is regulated by two VEGF-dependent pathways: the VEGF–eNOS–soluble guanylate cyclase (sGC)–cGMP pathway and a VEGF-dependent, nitric oxide-independent pathway[211,212]. A study in cirrhotic rats demonstrated that administration of an sGC activator that induces the VEGF–eNOS–sGC–cGMP pathway promoted the restoration of LSEC fenestration, leading to HSC quiescence and cirrhosis regression[211]. Oxidative stress related to nitric oxide signalling has been implicated in LSEC dysfunction, leading to fibrogenesis. For example, reduced nitric oxide bioavailability has been observed in LSECs isolated from cirrhotic rat livers[213]. Similarly, in a rat model of early-stage NAFLD, endothelial dysfunction was associated with reduced ER bioavailability and increased cyclooxygenase end products and oxidative stress[214].

Additional studies have aimed to identify regulators of nitric oxide bioavailability in LSECs. For example, a study by Su and colleagues utilized single-cell RNA sequencing of normal and cirrhotic mouse livers to identify the spatial distribution of heterogeneous LSECs[215]. This study demonstrated that transcription factors (KLF2, KLF4 and AP-1) that induce nitric oxide production in response to shear stress were downregulated in LSECs of all zones in cirrhotic mice[215]. A study in mice by Matsumoto and colleagues suggested that upregulation of NADPH oxidase, NOX1, in LSECs reduced the bioavailability of nitric oxide, leading to peroxynitrite-mediated cellular injury and impaired microcirculation[216]. Yang and colleagues explored alcohol-induced LSEC dysfunction and found that alcohol metabolism by CYP2E1 increased HSP90 acetylation and decreased its interaction with eNOS, leading to a decrease in nitric oxide production in mice. Overexpression of HDAC6 in LSECs deacetylated HSP90, restored the interaction between HSP90 and eNOS, and reduced alcohol-induced liver injury[217].

Others have implicated autophagy as a critical regulator in protecting LSECs from oxidative stress. Loss of endothelial autophagy leads to reduced intrahepatic nitric oxide, impaired ability of LSECs to handle oxidative stress and fibrosis amplification in the context of CCl_4-induced mouse liver injury[218]. Similarly, deficiency in endothelial autophagy induced inflammation, liver cell apoptosis and perisinusoidal fibrosis in a mouse model of NASH[219].

Macrophages

Endoplasmic reticulum stress. Several studies have highlighted a role of ER stress in macrophages. In the liver, there are two major populations of macrophages: the liver-resident Kupffer cells and the monocyte-derived macrophages[220]. A study by Wang and colleagues showed that ER stress in Kupffer cells promoted apoptotic clearance of activated HSCs and the resolution of hepatic fibrosis in rats. Mechanistically, the researchers observed that ER stress in Kupffer cells promoted polarization towards the M1 phenotype, increasing TNF expression levels. The increase in TNF promoted HSC apoptosis through TNF-R1–caspase 8 axis[221].

Others identified Nogo-B (also known as reticulon 4B), an ER-resident protein that regulates ER structure and function, as a potential target in Kupffer cells for the treatment of ALD[222]. A positive correlation was observed between Nogo-B-positive Kupffer cells and disease severity in patients with ALD, and *Nogo-B*-knockout mice showed decreased liver injury in response to chronic ethanol feeding[222]. In Kupffer cells, the absence of *Nogo-B* increased ER stress and M2 polarization[222].

Wang and colleagues investigated the role of XBP1 in regulating macrophage STING signalling and liver fibrosis. They observed increased XBP1 expression in fibrotic liver macrophages in mice and patients, and demonstrated that myeloid-specific *Xbp1* deficiency reduced fibrosis development[223]. The authors showed that XBP1 regulated macrophage cGAS–STING–NLRP3 activation by modulating self-mtDNA cytosolic leakage. Their findings suggest that targeting XBP1 signalling in macrophages might represent a novel antifibrotic strategy[223].

Oxidative stress. In response to stimuli such as alcohol and endotoxins, Kupffer cells release mediators to adjacent cells, including hepatocytes and HSCs. Kupffer cells have a central role in ROS generation via the activity of NOX2 in association with TLR signalling[224]. Kim and colleagues observed in mice that Kupffer cells generate ROS via TLR4 and NOX2, which stimulates the production of pro-inflammatory cytokines such as TNF, IL-6, and IL-1β[225]. Nieto demonstrated intercellular communication via ROS in a co-culture model of Kupffer cells and HSCs. They observed that Kupffer cells mediated a signalling cascade of ROS, IL-6 and phosphorylated p38 to regulate matrix degradation[226]. Wu and colleagues observed that induction of liver fibrosis with CCl_4 in mice was associated with increased expression of T cell immunoglobulin domain and mucin domain 4 (TIM4, also known as TIMD4) in Kupffer cells. Depletion of *Tim4* in Kupffer cells suppressed ROS production, mitophagy and TGFβ secretion, which led to decreased HSC activation and fibrosis attenuation[227].

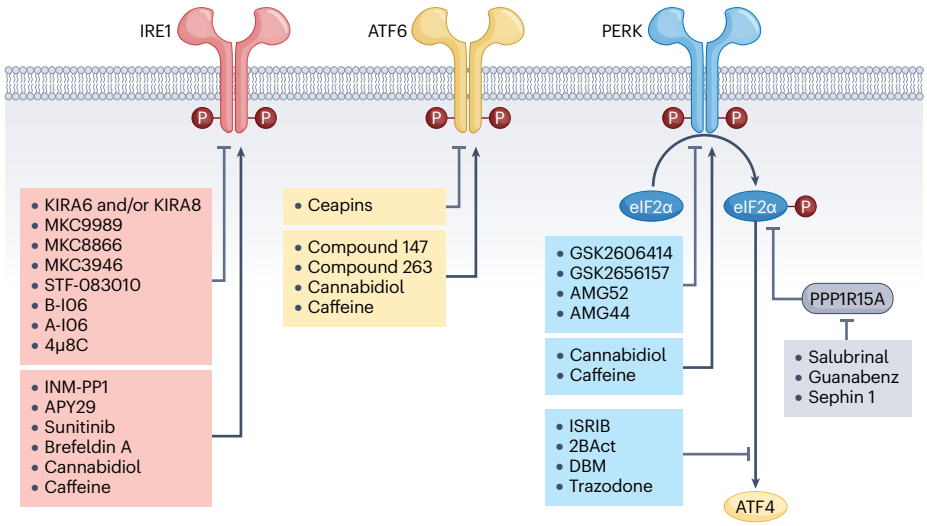

Fig. 4 | Small molecules targeting each UPR branch. The unfolded protein response (UPR) is activated in response to the endoplasmic reticulum (ER) stress and consists of three ER transmembrane protein stress sensors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase R-like endoplasmic reticulum kinase (PERK). Compounds that have been shown to regulate each of the three UPR branches are displayed. ATF4, activating transcription factor 4; PPP1R15A, protein phosphatase 1 regulatory subunit 15A. Adapted with permission from Wolters Kluwer Health, Inc.: Ajoolabady, A. et al. Endoplasmic reticulum stress in liver diseases. *Hepatology* **77**(2), 619–639 (https://journals.lww.com/hep/pages/default.aspx)[24].

Pyroptosis and iron homeostasis. Liver macrophages, in particular Kupffer cells, are a primary site of NLRP3 inflammasome activation and a source of the pro-inflammatory cytokine IL-1β[228]. NLRP3 inflammasome-dependent pyroptosis in Kupffer cells has been implicated in liver fibrosis progression. In human fibrotic liver tissues, enhanced expression of NLRP3 compared with that in healthy controls was mainly localized to hepatocytes but also Kupffer cells, indicating that Kupffer cells also undergo pyroptosis[228]. These findings were validated in a CCl_4-induced mouse model, in which activation of NLRP3 mainly occurred in Kupffer cells[228]. Mechanistically, the investigators suggested that the activation of TLR4-dependent NF-κB signalling by DAMPs led to an increase in ROS levels and facilitated pyroptosis in macrophages, contributing to the progression of liver fibrosis[228]. He and colleagues investigated the mechanism of NLRP3 inflammasome in a mouse model of NAFLD and found that FFAs elevated the mitochondrial ROS in Kupffer cells and this subsequently induced mtDNA release to the cytoplasm. Released mtDNA activated the NLRP3 inflammasome and promoted IL-1β secretion in Kupffer cells[229,230]. Kaufmann and colleagues found that myeloid-specific NLRP3 inflammasome activation was crucial for NAFLD progression to fibrotic NASH. In a model in mice fed a HFD, myeloid-specific *Nlrp3*-knockout mice showed reduced inflammation and fibrosis and decreased HSC activation[231]. These studies highlighted the importance of macrophage NLRP3 inflammasome-dependent pyroptosis resulting in the activation of HSCs and liver fibrosis.

With respect to iron, Handa and colleagues demonstrated that iron disrupts the homeostasis between M1 and M2 macrophage polarization that led to increased fibrogenesis in a mouse model of NAFLD[232]. Similarly, Kanamori and colleagues demonstrated that iron-rich Kupffer cells displayed pro-inflammatory and profibrotic phenotypic changes during the development of NASH, as shown in a mouse model of NASH in which iron chelation treatment attenuated liver fibrosis[233].

Potential therapeutics to alleviate cellular stress and reduce fibrogenesis

Given the data suggesting that cellular stress has a marked role in fibrogenesis, including in the setting of NAFLD and NASH progression, strategies to target these pathways might have a substantial benefit in patients with chronic liver disease. Figure 4 lists compounds that modulate each of the three UPR branches and therefore have the potential to alleviate ER stress[24].

Conclusion

In summary, studies have demonstrated a critical role of cellular stress pathways in the pathogenesis of liver disease, particularly NAFLD and NASH, and fibrogenesis. Although the majority of studies have focused on hepatocytes, emerging studies in other cell types, including HSCs, have also emphasized that targeting cellular stress might be a useful therapeutic strategy to ameliorate fibrogenesis. Additional studies are warranted to identify novel cell-specific regulators of stress pathways to develop targeted therapeutic strategies. Furthermore, it is important to evaluate the effect of compensatory mechanisms that occur following the inhibition of an individual stress pathway. Many studies have utilized animal models to explore the effect of cellular stress, and additional studies to confirm the findings using human samples are needed. Similarly, understanding the efficacy and safety of strategies that target stress pathways in combination will be useful. It should be emphasized that fibrogenesis occurs over decades in patients with chronic liver disease, and potential therapeutics targeting cellular stress should avoid affecting adaptive UPR in other organs. Finally, as many patients have advanced fibrosis at the time of initial presentation, and strategies to reverse fibrosis are needed, additional studies on the effects of cellular stress pathways in the context of fibrosis regression are required.

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Author contributions

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